

First Named Inventor	: Michael E. Spurlock	
Appln. No.	: 09/928,522	
Filed	: August 13, 2001	Group Art Unit: 1647
Title	: Bovine Leptin Protein, Antisense and Antibody	Examiner: C. J. Saoud
Docket No.	: LL31.12-0015	

EXHIBIT A  
OF  
AMENDMENT AFTER FINAL  
FILED ON OCTOBER 6, 2006

Joseph Sambrook, Edward F. Fritsch, Thomas Maniatis, entitled  
Molecular Cloning, A Laboratory Manual  
Volume II, pp 8.45, 11.45-11.47, and 11.55-11.57  
Second Edition (1989)

BEST AVAILABLE COPY

**Molecular  
Cloning**

A LABORATORY MANUAL  
SECOND EDITION

All rights reserved  
© 1989 by Cold Spring Harbor Laboratory Press  
Printed in the United States of America

9 8 7 6 5 4 3 2 1

*Book and cover design by Emily Harste*

*Cover:* The electron micrograph of bacteriophage  $\lambda$  particles stained with uranyl acetate was digitized and assigned false color by computer. (Thomas R. Broker, Louise T. Chow, and James I. Garrels)

*Cataloging in Publications data*

Sambrook, Joseph

Molecular cloning : a laboratory manual / E.F.

Fritsch, T. Maniatis—2nd ed.

p. cm.

Bibliography: p.

Includes index.

ISBN 0-87969-309-6

1. Molecular cloning—Laboratory manuals. 2. Eukaryotic cells—Laboratory manuals. I. Fritsch, Edward F. II. Maniatis, Thomas III. Title.

QH442.2.M26 1987

574.87'3224—dc19

87-35464

Researchers using the procedures of this manual do so at their own risk. Cold Spring Harbor Laboratory makes no representations or warranties with respect to the material set forth in this manual and has no liability in connection with the use of these materials.

Authorization to photocopy items for internal or personal use, or the internal or personal use of specific clients, is granted by Cold Spring Harbor Laboratory Press for libraries and other users registered with the Copyright Clearance Center (CCC) Transactional Reporting Service, provided that the base fee of \$0.10 per page is paid directly to CCC, 21 Congress St., Salem MA 01970. [0-87969-309-6/89 \$00 + \$0.10] This consent does not extend to other kinds of copying, such as copying for general distribution, for advertising or promotional purposes, for creating new collective works, or for resale.

All Cold Spring Harbor Laboratory Press publications may be ordered directly from Cold Spring Harbor Laboratory, Box 100, Cold Spring Harbor, New York 11724. Phone: 1-800-843-4388. In New York (516)367-8423.

**IDENTIFICATION OF cDNA CLONES OF INTEREST****Methods of Screening**

There are three methods to screen cDNA libraries for clones of interest:

- Nucleic acid hybridization
- Immunological detection of specific antigens
- Sib selection either by hybrid selection and translation of mRNA or by production of biologically active molecules

Most cloning projects today are aimed at isolating cDNAs corresponding to rare mRNAs and therefore require screening of large numbers of recombinant clones. This can be carried out effectively with only two types of reagents: antibodies and nucleic acid probes. In those rare instances when both types of reagents are available, nucleic acid probes are preferred because they can be used under a variety of different stringencies that minimize the chance of undesirable cross-reactions. Furthermore, nucleic acid probes will detect all clones that contain cDNA sequences, whereas antibodies will react only with a subset of these clones (in some cases one in six at best) in which the cDNA has been inserted into the vector in the correct reading frame and orientation. cDNA libraries that are to be screened by antibodies therefore need to be larger (by a factor of at least 6) than those that are to be screened by nucleic acid probes. Consequently, when using antibody probes to search for a cDNA clone corresponding to a mammalian mRNA present at the level of 1 molecule/cell or less, it is desirable to construct cDNA expression libraries that contain in excess of  $10^7$  members. This is not easy, especially when the amounts of mRNA are limited. Furthermore, screening a library of this size is expensive and laborious, and it becomes worthwhile to explore methods to enrich the mRNA (or cDNA derived from it) for the sequences of interest (see pages 8.6–8.10).

**NUCLEIC ACID HYBRIDIZATION**

This is the most commonly used and reliable method of screening cDNA libraries for clones of interest. None of the other methods displays such an abundance of attractive features. Screening by nucleic acid hybridization allows extremely large numbers of clones to be analyzed simultaneously and rapidly, does not require that the cDNA clones be full-length, and does not require that an antigenically or biologically active product be synthesized in the host cell. Furthermore, as a result of more than 20 years of work, the theoretical basis of nucleic acid hybridization is well-understood. This has led to the development of a large number of different techniques that can accommodate nucleic acid probes of very different lengths and specificities. Details of the methods for the preparation and use of these probes are presented in Chapters 10 and 11.

*Homologous probes*

Homologous probes contain at least part of the exact nucleic acid sequence of the desired cDNA clone. They are used in a variety of circumstances, for

---

## ***Conditions for Hybridization of Oligonucleotide Probes***

---

When using oligonucleotides as probes, the aim is to find conditions that are stringent enough to guarantee specificity and sufficiently flexible to allow formation of stable hybrids at an acceptable rate. For DNA molecules more than 200 nucleotides in length, hybridization is usually carried out at 15–25°C below the calculated melting temperature ( $T_m$ ) of a perfect hybrid. However, as the length of the probe is decreased, the  $T_m$  is lowered to the point where it is often impractical to carry out hybridization at  $T_m - 25^\circ\text{C}$ . Typically, therefore, hybridization with synthetic oligonucleotides is carried out under conditions that are only 5–10°C below the  $T_m$ . Although such stringent conditions reduce the number of mismatched clones that are detected with short oligonucleotide probes, they have the less desirable consequence of reducing the rate at which perfect hybrids form.

Hybrids formed between DNA molecules more than 200 nucleotides in length are completely stable for all practical purposes. The chances that such a long stretch of double helix will unwind at temperatures 15–25°C below the  $T_m$  are extremely small. However, hybrids (even perfect hybrids) formed between short oligonucleotides and their target sequences at 5–10°C below the  $T_m$  are far easier to unwind, and hybridization reactions of this type can be regarded as reversible. This has important practical consequences. Whereas hybrids formed between longer DNA molecules are essentially stable under the conditions used for posthybridization washing, hybrids (even perfect hybrids) involving short oligonucleotides are not. Posthybridization washing of such hybrids must therefore be carried out rapidly so that the probe does not dissociate from its target sequence. For this reason, hybridizations with short oligonucleotides should be carried out under stringent conditions (5–10°C below the  $T_m$ ) using high concentrations (0.1–1.0 pmole/ml) of probe. When only one or a small number of oligonucleotides (<8) are used as probes, the annealing reaction rapidly reaches equilibrium, and hybridization should therefore be terminated after 3 or 4 hours. More complex mixtures, in which the concentration of each oligonucleotide is comparatively low, require hybridization to be carried out for proportionately longer periods. For example, mixtures of 32 or more oligonucleotides should be hybridized for 1–2 days. Posthybridization washing should be carried out for brief periods of time, initially under conditions of low stringency and then under conditions of stringency equal to those used for hybridization.

***CALCULATING MELTING TEMPERATURES FOR PERFECTLY MATCHED HYBRIDS BETWEEN OLIGONUCLEOTIDES AND THEIR TARGET SEQUENCES***

When using single oligonucleotides that match the target sequence perfectly, hybridization conditions can easily be derived from the calculated  $T_m$  of the hybrid. For oligonucleotides shorter than 18 nucleotides, the  $T_m$  of the hybrid can be estimated by multiplying the number of A + T residues in the hybrid by 2°C and the number of G + C residues by 4°C and adding the two numbers (Itakura et al. 1984). However, this method overestimates the  $T_m$  of hybrids involving longer oligonucleotides.

A different approach has been taken by E. Fritsch (unpubl.), who found that the equation originally used to calculate the relationship between G + C content, ionic strength of the hybridization solution, and the  $T_m$  of long DNA molecules (Bolton and McCarthy 1962):

$$T_m = 81.5 - 16.6(\log_{10}[\text{Na}^+]) + 0.41(\% \text{ G} + \text{C}) - (600/N),$$

where  $N$  = chain length, predicts reasonably well the  $T_m$  for oligonucleotides as long as 60–70 nucleotides and as short as 14 nucleotides.

**ESTIMATING THE EFFECTS OF MISMATCHES**

Perhaps surprisingly, the classic formula (Bonner et al. 1973) to calculate the effect of mismatches on the stability of long DNA hybrids holds reasonably well for hybrids involving short oligonucleotides: For every 1% of mismatching of bases in a double-stranded DNA, there is a reduction of  $T_m$  by 1–1.5°C. However, the precise effect of mismatches depends on the G + C content of the oligonucleotide and, even more critically, on the distribution of mismatched bases in the double-stranded DNA. Mismatches in the middle of the oligonucleotide are far more deleterious than mismatches at the ends. Therefore, the method of estimation given above should only be used as a rough guide until a systematic study of all types of mismatches in a variety of contexts leads to more precise methods of estimation. If appropriate target DNA has been cloned, the effect of mismatches on  $T_m$  can be determined empirically (see pages 11.55–11.57).

**EMPIRICAL DETERMINATION OF MELTING TEMPERATURE**

The melting temperature ( $T_m$ ) of an oligonucleotide hybridized to a target sequence can be determined by the procedure described below. The protocol actually measures the temperature at which dissociation of the double-stranded DNA becomes irreversible ( $T_i$ ) in nonequilibrium conditions that do not favor rehybridization of the released probe to the target. The optimal temperature for hybridization is then determined on the basis of this value. The procedure requires a cloned target sequence that is complementary (perfectly or imperfectly, depending on the experiment) to the oligonucleotide probe. In most cases, a target sequence is not available from "natural" sources and must be synthesized chemically. The best synthetic target sequences consist of two oligonucleotides that are partially complementary. After annealing, these oligonucleotides form a double-stranded region that contains the target sequence. The sequences of the protruding ends are designed to allow the target DNA to be cloned easily in bacteriophage M13 vectors. Single-stranded DNA of the appropriate orientation prepared from the resulting clones (see Chapter 4) can be used in hybridization experiments as described below. It can also be used as a template for dideoxy-mediated chain-termination sequencing (see Chapter 13) if it is necessary to check that the sequence of the target DNA is correct.

1. Label 1–10 pmoles of the oligonucleotide to be used as a probe by phosphorylation (see pages 11.31–11.32), and remove excess unincorporated [ $\gamma$ - $^{32}$ P]ATP by one of the methods described on pages 11.33–11.39.
2. Using a paper punch, prepare four small circles (diameter 3–4 mm) of a solid support (nitrocellulose filter or nylon membrane) used for hybridization. Arrange the small circles on a piece of Parafilm. Mark two of the filters with a soft-lead pencil.
3. Apply approximately 100 ng of target single-stranded DNA in a volume of 1–3  $\mu$ l of  $2 \times$  SSC to each of the marked filters. Apply an equal amount of vector DNA to the unmarked filters. After the fluid has dried, use blunt-ended forceps (e.g., Millipore forceps) to remove the two sets of filters from the Parafilm, and place them between sheets of Whatman 3MM paper. Fix the DNAs to the filters by baking for 1–2 hours at 80°C in a vacuum oven.

If the target DNA has been cloned into a plasmid, linearize the vector by digestion with a restriction enzyme that does not cleave within the target sequences. Purify the resulting double-stranded DNA by extraction with phenol:chloroform and precipitation with ethanol. Dissolve the DNA in  $2 \times$  SSC at a concentration of 500 ng/ $\mu$ l. Apply the solution of DNA to the filters prepared as described above, and then, using blunt-ended forceps, transfer the filters to a sheet of 3MM paper saturated with denaturing solution (1.5 M NaCl, 0.5 N NaOH) for 5–10 minutes. Move the filters to a fresh sheet of 3MM paper saturated with neutralizing solution (0.5 M Tris · Cl [pH 7.4], 1.5 M NaCl) for 10 minutes. Transfer the filters to a dry sheet of 3MM paper, and leave them at room temperature until all of the fluid has evaporated. Bake the filters as described above.

Overbaking can cause the filters to become brittle. In addition, filters that have not been completely neutralized turn yellow or brown during baking and chip very easily. The background of nonspecific hybridization also increases dramatically.

4. Using blunt-ended forceps, transfer all of the filters to a polyethylene tube that contains 2 ml of oligonucleotide prehybridization solution. Seal the tube and incubate, with occasional shaking, at a temperature estimated to be  $T_m - 25^\circ\text{C}$  for the solvent being used (see Note i). After 2 hours, add radiolabeled oligonucleotide to the prehybridization solution. The final concentration of oligonucleotide should be approximately 1 pmole/ml. Continue incubation at  $T_m - 25^\circ\text{C}$  for a further 2–4 hours, with occasional shaking.

*Oligonucleotide prehybridization solution*

6 × SSC (or 6 × SSPE)  
 0.01 M sodium phosphate (pH 5.8)  
 1 mM EDTA (pH 8.0)  
 0.5% SDS  
 100 µg/ml denatured, fragmented salmon sperm DNA (see Appendix B)  
 0.1% nonfat dried milk

5. Remove the filters from the hybridization solution, and immediately immerse them in 2 × SSC at room temperature. Agitate the fluid continuously. Replace the fluid every 5 minutes until the amount of radioactivity on the filters remains constant (as measured with a hand-held minimonitor).
6. Adjust the temperature of a circulating water bath to  $T_m - 25^\circ\text{C}$ . Dispense 5 ml of 2 × SSC into each of 20 glass test tubes (17 mm × 100 mm). Monitor the temperature of the fluid in one of the tubes with a thermometer. Incubate the tubes in the water bath until the temperature of the 2 × SSC is  $T_m - 25^\circ\text{C}$ . The 2 × SSC in each of these tubes will be used separately for each temperature increase (see steps 7–10).
7. Transfer the filters individually to four empty glass tubes, separating the filters containing the vector and target DNAs, and add 1 ml of 2 × SSC (from one of the tubes prepared in step 6 and prewarmed to  $T_m - 25^\circ\text{C}$ ). Place the tubes in the water bath for 5 minutes.
8. Remove the tubes from the bath, transfer the liquid to scintillation vials, and wash the tubes and filters with 1 ml of 2 × SSC at room temperature. Add the wash solutions to the appropriate scintillation vials.
9. Increase the temperature of the water bath by  $3^\circ\text{C}$ , and wait for the temperature of the 2 × SSC in the tubes prepared in step 6 to equilibrate.
10. Add 1 ml of 2 × SSC at the higher temperature to each of the four tubes containing the filters. Place the tubes in the water bath for 5 minutes.



11. Repeat steps 8, 9, and 10 at successively higher temperatures until a temperature of  $T_m + 30^\circ\text{C}$  is achieved.
12. Place the filters in separate glass tubes (17 mm  $\times$  100 mm) containing 1 ml of  $2 \times \text{SSC}$ , and heat them to boiling for 5 minutes to remove any remaining radioactivity. Cool the solutions in ice, and transfer them to scintillation vials. Wash the filters and tubes used for boiling with 1 ml of  $2 \times \text{SSC}$ , and add the washing solutions to the appropriate scintillation vials.
13. Use a scintillation counter to measure the radioactivity (by Cerenkov counting, see Appendix E) in all of the vials. Calculate the proportion of the total radioactivity that has eluted at each temperature (i.e., the sum of radioactivity eluted at all temperatures between  $T_m - 25^\circ\text{C}$  and the temperature at which a given sample was taken divided by the total radioactivity eluted from the filters at all temperatures up to and including  $100^\circ\text{C}$ ).

If the experiment has worked well, very little radioactivity should be associated with the filters containing vector DNA alone. Furthermore, this radioactivity should be completely released from the filters at temperatures much lower than the estimated  $T_m$ . On the other hand, considerable radioactivity should be associated with the filters containing the target DNA; the elution of this radioactivity should show a sharp temperature dependence. Very little radioactivity should be released from the filters until a critical temperature is reached, and then approximately 90% of the radioactivity should be released during the succeeding 6–9°C rise in temperature. The temperature at which 50% of the radioactivity has eluted from the filters containing the target sequences is defined as the  $T_i$  of the hybrid between the probe and its target sequence.

## Notes

- i. Although the above protocol calls for the use of sodium salts in the solvent used for hybridization, other solutes such as tetramethylammonium chloride or tetraethylammonium chloride can be substituted if desired to determine the  $T_i$  in these solvents.
- ii. This method can easily be adapted to study the behavior of hybrids formed between probes and target sequences that do not match each other perfectly (Jacobs et al. 1988).
- iii. Before synthesizing the probe, check for potential homology and/or complementarity between its sequence and the sequence of the vector used to propagate the target. Most of the commercially available programs to analyze DNA can be used to search commonly used vectors for sequences that match the sequence of the probe closely enough to cause problems during hybridization.